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Microbial combinatorics: a simplified approach for isolating insecticidal bacteria

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Bacteria can control pest insects that damage food crops, vector diseases and defoliate trees. Conventionally, isolation of these bacteria has been from soil and sporadically from dead insects. A simplified approach for isolating insecticidal bacteria from soil using the target insect as the selective agent was employed in this study. Instead of isolating single strains of bacteria from soil and testing each individual strain for insect toxicity, mixtures of bacteria present in each soil sample were tested together directly for toxicity using *Manduca sexta* (Linnaeus) (Lepidoptera: Sphingidae) as a model insect. Thirty-five soil suspensions or bacterial suspensions of the 40 suspensions tested killed at least one *M. sexta* larva. All but one bacterial culture isolated from dead larvae and retested for toxicity, killed at least one *M. sexta* larva. Nineteen bacterial strains isolated from larvae killed in the first test, were identical to the bacteria fed to the retested larvae. Of the 19 strains isolated, 14 were identified by 16S rDNA sequencing as belonging to the *Bacillus cereus* group including three strains that formed crystals that were identified as *B. thuringiensis*. Of the three other spore-forming strains, two were identified as psychrotrophic *B. weihenstephanensis* and the third as *Lysinibacillus fusiformis*. Two others were identified as *Enterococcus faecalis*. This approach, microbial combinatorics, reduces the number of insects necessary for toxicity screening and associated time and resources compared to conventional methods that first isolate bacteria and then individually test for toxicity as well as a means of discovery of new pathogens using the insect as the selective agent.

Keywords: *Bacillus thuringiensis; B. weihenstephanensis; Lysinibacillus fusiformis;* insect pathogens; *Manduca sexta*

Introduction

The demand for organic products and the development of resistance of insects to conventional pesticides as well as biological pesticides (Griffitts and Aroian 2005) has led us to search for additional environmental bacteria that are toxic to insect pests. In the field of insect pathology, conventional screening methods use insects to determine toxicity of previously isolated bacteria. This process often starts with plating environmental samples, identifying the bacteria, and then testing selected bacteria for toxicity to insects. Since soil can contain more than 10^9 colony-forming units (cfu) per gram, few of which are toxic to insects (Martin and Travers 1989), both labour and supply costs to test each bacterium can be formidable. An

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alternative to the conventional method of screening borrows a concept from combinatorial chemistry (Borman 2000) in which compounds are tested in groups for the desired effect. Initial groups which do not demonstrate the desired effect are eliminated from further testing. We have previously demonstrated that mixtures of strains of bacteria can be screened in combination for toxicity to insects (Martin and Blackburn 2007).

Since soil contains a natural mixture of potentially insecticidal microbes, we tested mixed cultures from soils for their ability to kill insects, using *Manduca sexta* L. (Lepidoptera: Sphingidae) as a model insect (Silva et al. 2002). Soil suspensions were tested directly for toxicity. In addition, the bacteria in these soils were grown *in vitro* to increase the concentration of potentially pathogenic bacteria that may be present in soil at too low a concentration to kill an insect. This step also distinguishes the direct killing of an insect by a residual pesticide present in the soil from that of a pathogen.

Pathogens such as *Bacillus thuringiensis* Berliner, have been isolated from the soil and are known to kill insects (Ohba and Aizawa 1986; Travers, Martin, and Reichelderfer 1987). If *M. sexta* larvae died after feeding on bacteria grown from soil, bacteria were isolated from the dead larvae. To demonstrate that a bacterium recovered from the dead larva was responsible for the death of the insect, a pure culture was retested for ability to kill the same type of insect (Koch's postulates as per Black 1996). This simplified approach, microbial combinatorics, for selecting and testing prospective insect pathogens *in vivo*, i.e. use of the insect to directly isolate pathogenic bacteria from mixtures rather than testing bacteria previously isolated and purified, was effective. In most cases, the larvae actually purified and amplified the bacteria responsible for death in a single step.

Many of the crystal forming *B. thuringiensis* strains isolated by other methods (Ohba and Aizawa 1986, Travers et al. 1987) are not toxic to insects (Martin and Travers 1989). Since the ultimate goal in isolating bacterial strains is identifying strains toxic to insects, it is reasonable that an insect be used to screen environmental samples for pathogenic bacteria. Many toxic *B. thuringiensis* strains were originally isolated from dead insect larvae (silk worms, Ishiwata 1901, or flour moths, Berliner 1915) and toxic strains have been isolated from outbreaks in rearing facilities (Dulmage 1970; Itoua-Apoyolo et al. 1995).

Materials and methods

Bacterial strains and media

Bacillus thuringiensis strain IBL 455, originally isolated from a 1981 preparation of Dipel (Abbott Laboratories, Chicago, IL) was used as a positive control to kill *M. sexta* larvae. Another *B. thuringiensis* strain, IBL 717, that was not toxic to *M. sexta* was used as a control for bacterial load. Bacteria were grown on L-agar (10 g tryptone, 5 g yeast extract, 5 g NaCl, 10 g agar/L, Atlas 2004) or RM (½ L-agar) for enumeration and recovery and incubated at 25°C for 48 h. If crystals were detected, bacteria were grown on T3 (tryptone, tryptose, yeast extract, phosphate buffer (pH 6.8) $MgSO_4$, MnCl, Travers et al. 1987) to obtain maximum crystal production for insect assays.

Biochemical media were used as described for *Bacillus* (Parry, Turnball, and Gibson 1983). Fourteen different media were used to test for various phenotypic traits including acid production from glucose, arabinose, xylose, mannitol, mannose, cellobiose, salicin, and sucrose. In addition, utilisation of citrate and esculin; and production of secondary metabolites: protease, amylase, urease, phosopholipase C, and hemolysin were also determined. Antibiotic sensitivities were also compared using antibiotic discs (Becton-Dickinson, Sparks, MD) with ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin, tetracycline, triple sulfa, and vancomycin.

Soil samples

Twenty soil samples were collected along Fishing Creek in the City of Frederick (MD) Municipal Forest in July 2004 because bacteria toxic to insects were previously isolated from this area (Martin, Blackburn, and Shropshire 2004). Storage for 1 year at ambient temperature in sealed bags favoured isolation of spore-forming bacteria. Moisture content of the soils when tested ranged from 3.1 to 68.8% as determined by drying a known weight of soil in a convection oven at 64°C until the weight did not change. Soil was suspended 1:10 (w:v) in sterile distilled water, shaken on a vortex mixer for 30 min, then plated immediately on RM for initial enumeration of bacteria. An undiluted sample (0.1 mL) of this soil suspension was plated on L-agar and incubated at 25°C for 24 and 48 h for toxicity testing. The remaining soil suspension was incubated under the same conditions to be tested in parallel with the bacteria grown from the same sample. The soil suspension and the bacterial suspension grown from the soil were each fed to *M. sexta* larvae. Bacteria recovered from soil suspensions after incubation or harvested after growth were enumerated on RM.

Insect bioassays

For bioassays, tobacco hornworm diet containing wheat germ, sucrose, casein as well as salts, vitamins and acids to prevent fungal growth (THW diet, Martin and Blackburn 2007) was prepared as re-hydrated freeze-dried pellets (Martin 2004). The molten diet was poured into 96-well polypropylene plates (GreinerBioOne, Longwood, FL), frozen overnight (-20°C), and then dried in a Virtis Advantage Freeze Dryer (Virtis Co., Inc., Gardiner, NY). The dry THW diet pellets were removed from the 96-well plates, placed in sterile plastic bags, and stored at 4°C until use.

Manduca sexta eggs were received from J. Pennington (U. Arizona) and reared on THW diet at 24°C, 46% RH, and 16:8 light:dark cycle until second instar. Diet was changed every 2–4 days.

Sixteen diet pellets were used for each treatment in bioassays with one diet pellet placed in a well (1.6 $\times$ 1.6 cm, diameter $\times$ deep) in white plastic bioassay trays (C-D International, Ocean City, NJ). Pellets were re-hydrated with 0.3 mL of sterile distilled water (controls) or suspensions containing extracts of soil or dilutions of bacteria grown on plates which were enumerated by serial dilution on RM. One *M. sexta* second instar larva was added to each well. Wells were sealed with bioassay tray covers (C-D International, Ocean City, NJ) and holes made in the covers with insect pins for oxygen transfer. Larvae were incubated as for rearing. Mortality was

recorded at 16, 24, 48, 72, 96 and 120 h. Controls were done for every five soil suspensions or bacterial suspensions screened.

For LC_{50}s, 32 insects and at least five doses were used for toxicity assays. Dose was determined by protein concentration on whole cultures using Bradford reagent (Sigma Chemical Co., St. Louis, MO) for crystal-forming bacteria that had been harvested in sterile water from T3 plates after sporulation.

Recovery of bacteria from larvae

From each treatment with larval mortality, at least one dead larva was removed to recover bacteria. To show that the bacteria recovered from a dead larva were responsible for larval death, we used Koch's postulates (Black 1996). Second instar larvae that had died following treatment with bacteria or soil were surface sterilised by dipping for 3 s in 10% bleach and rinsing in sterile distilled water, placed in a sterile plastic bag, suspended in 5 mL sterile distilled water, and ground in a stomacher blender (Techmar, Cincinnati, OH) for 60 s on high. The larval extract and dilutions were plated on RM. Plates were incubated at 25°C for 48 h. Colonies were enumerated and the predominant colony type was isolated and checked for the formation of spores and crystals by phase-contrast microscopy and characterised by biochemical tests and sensitivity to antibiotics. A pure culture of these bacteria was grown on L-agar for spore-forming bacteria that did not make crystals or T3-agar (Travers et al. 1987) for spore-forming bacteria that made crystals. These bacteria were harvested after 48 h of growth and fed to second instar larvae as described above. Bacteria isolated from these dead larvae were compared to the bacteria originally isolated from the larvae by biochemical profiles (Martin, Haransky, Travers, and Reichelderfer 1985) and antibiotic sensitivities (Becton-Dickinson). A flow chart describing the procedure is shown in Figure 1.

Bacterial identification

Individual isolates were identified by PCR amplification and sequencing of conserved 16S ribosomal DNAs. For each isolate, DNA was purified from a 2-mL culture that was grown either overnight or for only 8 h (for strains that sporulated quickly) at 25°C at 250 rpm. DNA was isolated using the Quantum Prep miniprep kit (BioRad) as specified by the manufacturer for use as template in polymerase chain reaction (PCR). Nearly full length 16S rDNA was amplified for each isolate using primers universal to prokaryotes, R16F0 and R16R0 (Lee, Hammond, Davis, and Gundersen 1993). Thirty-five PCR cycles were conducted in a model 9700 thermocycler (Applied Biosystems) using 30 s denaturation at 94°C, 1.5-min annealing at 55°C, and 2-min primer extension (10-min in final cycle) at 72°C. Bacterial 16S rDNA amplicons were sequenced directly. Products were separated on 1.5% NuSieve agarose gel (FMC, Rockland, ME) in modified-1 × TAE (0.04 M Tris-acetate and 0.1 mM EDTA), and excised for sequencing using ABI BigDye V1.1 (Applied Biosystems with the amplification primers and a nested universal primer 533F (5′-GTGCCAGCMGCCGCGGTAA-3′). Cycle sequencing conditions were 35 cycles at 96°C, 10 s; 50°C, 5 s; 60°C for 4 min. Automatic sequencing was carried out on an ABI Prism Model 3100 (Applied Biosystems). Sequences were edited and assembled (DNASTAR, SeqMan component); BLAST (Altschul, Gish, Miller,

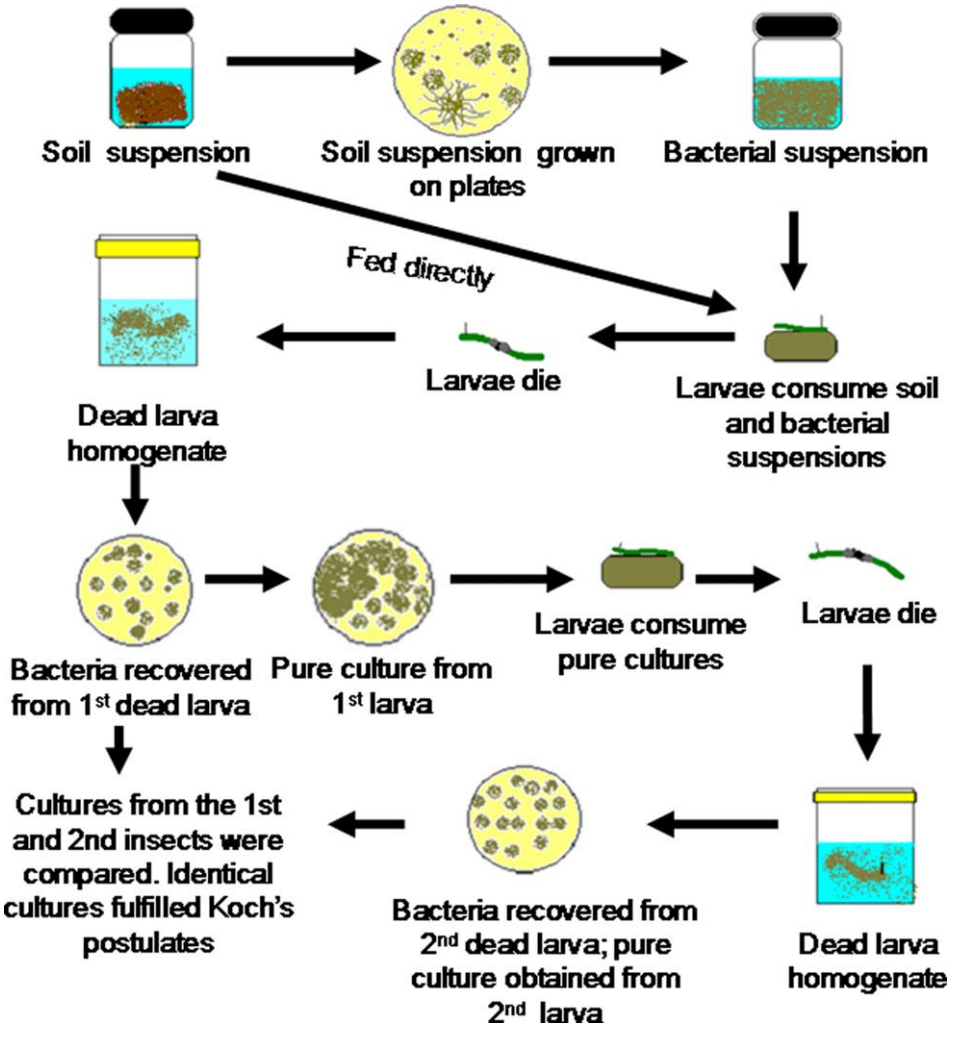

Figure 1. Flow chart describing isolation of insect pathogenic bacteria from soil.

Myers, and Lipman 1990) searches were conducted to identify bacterial isolates based on rDNA sequence homology to known bacteria. GenBank accession numbers for 16S sequences for each new bacterial isolate are given in Table 1.

Statistical analysis

To compare relative toxicities of mixed cultures of bacteria, survival analysis was done to determine speed of kill (SAS Inc. 2004). The LC_{50} was determined by probit analysis (SAS Inc. 2004) for each strain that killed 75% of the larvae using an undiluted sample. For those strains that formed crystals, dose was determined by measuring protein concentration of whole cultures (spores and crystals) produced by the sporulated bacteria using Bradford reagent (Sigma Chemical Co.). For strains that did not produce crystals, viable cell count was used as dose. Regression analysis

Table 1. Identification of bacteria isolated from *Manduca sexta* larvae with Genbank accession number for 16S rDNA sequence.

Soil No	Species identification	GenBank accession number 16S rDNA
18	*Bacillus thuringiensis*	EU168405
18S[a]	*Bacillus thuringiensis*	EU168406
8	*Bacillus thuringiensis*	EU168410
1	*Bacillus cereus*	EU168402
11	*Bacillus cereus*	EU168413
12	*Bacillus cereus*	EU168403
14	*Bacillus cereus*	EU168404
16	*Bacillus cereus*	EU168415
17	*Bacillus cereus*	EU168416
19	*Bacillus cereus*	EU168407
21	*Bacillus cereus*	EU168417
5S	*Bacillus cereus*	EU168408
7	*Bacillus cereus*	EU168409
9	*Bacillus cereus*	EU168411
11S	*Bacillus weihenstephanensis*	EU168412
5	*Bacillus weihenstephanensis*	EU168414
10	*Lysinibacillus fusiformis*	EU168418
17S	*Enterococcus faecalis*	EU168400
4	*Enterococcus faecalis*	EU168401

[a]S after sample designation indicates that the strain was isolated from larvae which had been fed soil suspensions rather than amplified bacterial suspensions.

was used to determine if there were correlations between concentrations of bacteria and survival. Mean weights of *M. sexta* larvae were compared by analysis of variance using the MIXED procedure (SAS Inc. 2004) and are reported as probabilities from *F*-test or *t*-tests.

Results

Initial toxicity

All 20 bacterial suspensions, grown 48 h to allow sporulation or secondary metabolism, killed greater than 33% of the larvae within 72 h (Table 2). Within 96 h, bacterial suspensions from 17 of the 20 soil samples killed all larvae. The titers from these bacterial suspensions averaged 1.00×10^7 cfu/diet pellet and ranged from 4.8×10^3 to 1.66×10^{10} cfu/diet pellet. While the larvae were fed various concentrations of bacteria per treatment depending on what grew from the soil, the concentration of bacteria fed was not correlated to speed of kill as measured by survival ($n = 17$, $r^2 = 0.33$; Figure 2).

Larvae fed bacterial suspensions that were grown on L-agar for only 24 h survived the 120 h of the experiment. All larvae fed a spore/crystal suspension of the control *B. thuringiensis* IBL 455 grown for 48 h on T3 died within 24 h (10^6cfu/diet pellet). There was no mortality in the water control.

Only 15 soil suspensions killed at least one *M. sexta* larva when fed directly. Bacterial counts averaged 4.59×10^5 cfu/diet pellet and ranged from 1.08×10^3 to

Table 2. *Manduca sexta* mortality fed soil or bacterial suspensions.

Soil No	Initial soil titer/g $\times 10^6$	Initial% mortality soil suspension	Fulfilled Koch's postulates	ID[a]	Initial bacterial titer/mL $\times 10^9$	Initial% mortality bacterial suspension	Fulfilled Koch's postulates	ID
1	0.04	0	No kill[b]		0.1	33	Yes	Bc
3	0.26	13.3	No kill		83	100	No	
4	0.05	12.5	No[c]		ND[d]	100	Yes	Ef
5	0.05	12.5	Yes[e]	Bc	0.002	100	Yes	Bw
6	10.2	8.3	No		0.32	100	No	
7	0.1	18.8	No		ND	100	Yes	Bc
8	0.02	33.3	No kill		5.4	100	Yes	Bt
9	21.0	0	No kill		0.06	100	Yes	Bc
10	0.49	12.5	No kill		2.0	100	Yes	Lf
11	0.004	6.3	Yes	Bw	3.6	100	Yes	Bc
12	0.26	6.3	No kill		1.7	100	Yes	Bc
13	6.2	0	No kill		0.51	100	No	
14	0.32	12.5	No kill		0.33	100	Yes	Bc
15	0.18	6.3	No kill		0.36	100	No kill	
16	0.11	18.8	No kill		3.7	100	Yes	Bc
17	0.23	12.5	Yes	Ef	0.001	81.4	Yes	Bc
18	0.05	18.5	Yes	Bt	0.5	100	Yes	Bt
19	0.03	6.3	No kill		0.05	100	Yes	Bc
20	17.1	0	No kill		0.3	75	No	
21	0.07	0	No kill		7.4	100	Yes	Bc
Bacterial control					1.2	0		
Avg	2.8	9.9			5.5	94.5		

Control mortality was 0 at 120 h for all but one bioassay (soil samples 6 and 20) in which control mortality was 6%. Soils were tested in batches of four to a control. [a]Abbreviations: Bc, *Bacillus cereus*; Bw, *Bacillus weihenstephanensis*; Ef, *Enterococcus faecalis*; Bt, *Bacillus thuringiensis;* Lf, *Lysinobacillus fusiformis;* Bacterial control, *B. thuringiensis* IBL 717. [b]No kill means that there was no mortality when the bacteria isolated from dead insects were fed to healthy insects or soil suspensions did not kill in the first place. [c]No means insects were killed, but the bacteria isolated from dead insects did not phenotypically match the bacteria fed. [d]ND, not determined. [e]Yes means insects were killed and the bacteria isolated from the dead insects phenotypically matched the bacteria fed.

3.00×10^6 cfu/diet pellet. The bacterial colonies on these plates ranged from spreading *B. mycoides* Smith and Couche (1991) types to a general slime, to *B. cereus* Frankland and Frankland type colonies, to large transparent colonies as well as several different sizes (from pinpoint to 2 mm) and shapes of cream-coloured colonies. Occasionally yellow, orange, or pink colonies were also present in the initial soil titers. Mortality for larvae fed soil suspensions directly was not greater than 33% for any soil tested (Table 2) and averaged 9.9% mortality compared to 94.5% mortality of insects fed bacterial suspensions.

Autoclaved soil suspensions (which had no viable bacteria when 0.1 mL of a 1-g/10-mL suspension) fed to second instar larvae did not kill any larvae. Water controls also had no mortality. To control for bacterial load, we fed *M. sexta* larvae a high dose of a non-toxic *B. thuringiensis* strain, IBL 717 and there was no mortality.

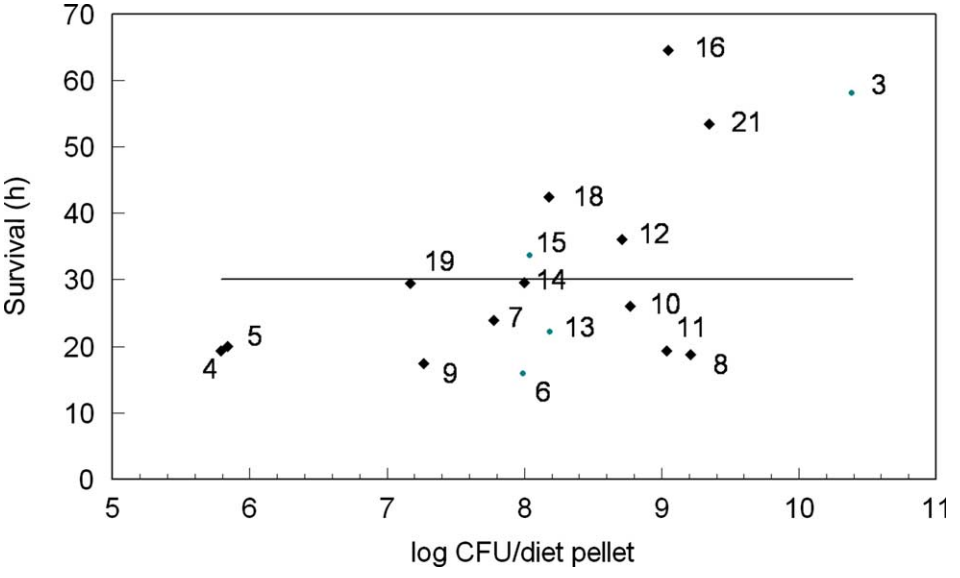

Figure 2. Survival times versus cell concentrations of bacterial suspensions from which the identified pathogens were isolated. Control mortality was 0%. The line is the average survival time for all bacterial suspensions tested. Samples are indicated by number. Diamonds are samples from which pathogens which fulfilled Koch's postulates were isolated. Filled squares are those that did not fulfill Koch's postulates.

Weights from surviving insects fed soil samples 4 (51.3 ± 5.2 mg) and 7 (56.0 ± 9.4 mg) were slightly higher, but not statistically significant (ANOVA df $=2$, $F=1.02$ $P=0.37$) from control weights (44.1 ± 4.5 mg).

Some of the larvae that died turned black, first around the midgut and eventually throughout the entire larva, a phenomenon typical of death by *B. thuringiensis* (Heimpel and Angus 1959). Other larvae that died turned brown from the posterior end, such as 1, 9, 11, and 21, while still others remained green and liquefied (5, 17).

Isolation of bacteria

Bacteria were recovered after plating on RM from at least one larva from each treatment with mortality. The concentration of bacteria recovered from these larvae averaged 2.97×10^7 and ranged from 6.12×10^6 to 9.95×10^7 cfu/larva. Most bacteria isolated from the same insect had the same colony morphology. Occasionally low numbers of Gram-negative bacteria were also co-isolated. Testing 32 colonies of the same morphology from a single soil sample showed that all 32 colonies gave identical results for 15 biochemical tests, to fulfill Koch's second postulate of a pure culture (as per Black 1996).

Pure cultures isolated from the dead larvae from the initial feeding were fed to healthy *M. sexta* larvae (Koch's third postulate). Twenty-five of 27 pure cultures killed at least one larva in this second assay. Four pure cultures that formed crystals killed 100% of the larvae. For two other bacterial cultures, the mortality of the pure culture increased from 33 to 50% (1) and from 12.5 to 37.5% (17S) as compared with the initial mixed culture. The bacteria isolated from this second assay were compared

to the pure cultures isolated from the corresponding insect assay. For 19 of 26 cultures, the 20 characteristics tested from the strains isolated in both the first and second assay were identical (Table 3 shows results of representative strains of the five species that killed larvae). Certain strains exhibited numerous differences, such as strains from soils 3, 6, 13 and 20. These differences confirmed that the selected biochemical tests could differentiate between closely related strains, fulfilling the final condition of Koch's postulates to show original and reisolated microbes were identical.

Identification of bacteria

Of the 19 strains that fulfilled Koch's postulates, 17 formed spores. Putative identities based on nearly full 16S rDNA showed that 14 belonged to the *B. cereus* species complex. Three also formed crystals (8, 18 and 18s) and were identified as *B. thuringiensis*. Two other spore formers, 11s and 5, were identified as *B. weihenstephanensis* Lechner et al., and like other strains of this psychrotolerant species, grew and formed spores at 4°C. A third spore forming strain, 10, formed spherical to oval spores that swelled the sporangia and was identified as *B. fusiformis* or, under a recent proposal, *Lysinibacillus fusiformis* (Ahmed, Yokota, Yamazoe, and Fujiwara 2007). The two non-spore forming bacteria were identified as *Enterococcus faecalis* Schleifer and Klipper-Balz and like other *E. faecalis* hydrolysed gelatin.

The three *B. thuringiensis* strains were different from each other and the commercial strain (IBL 455). The two strains that were isolated from soil sample 18 differed in the hydrolysis of esculin, and their sensitivity to neomycin. The

Table 3. Selected characteristics of representative bacterial species isolated from *Manduca sexta* larvae.

	Strain				
Characteristic	18	14	5	10	4
Acid from: Glucose	+	+	+	−	+
Salicin	+	+	+	−	−
Sucrose	−	−	+	−	−
Mannitol	−	−	−	−	+
Hydrolysis of Esculin	−	+	+	−	+
Production of: Urease	−	+	+	+	+
Protease	+	+	+	−	−
Amylase	+	+	+	−	−
Phospholipase C	+	+	+	−	−
Haemolysin	+	+	+	−	−
Sensitivity to: Ampicillin	R[a]	R	R	S	S
Kanamycin	I	S	I	S	I
Erythromycin	S	S	S	S	I
Presence of spores	+	+	+	+[b]	−
Presence of crystals	+	−	−	−	−

Only tests that differ among strains are shown. All strains shown did not produce acid from arabinose and xylose and were sensitive to tetracycline, vancomycin and chloramphenicol. 18, *B. thuringiensis*; 14, *B. cereus*; 5, *B. weihenstephanensis*; 10, *L. fusiformis*; 4, *E. faecalis*. [a]Response to the antibiotic: S, sensitive; R, resistant; I, intermediate. [b]Swollen sporangia.

B. thuringiensis strain from soil sample 8 also differed from the strain in Table 3 by three substrate tests (acid production from salicin, hydrolysis of esculin and production of urease). It also differed by intermediate resistance to tetracycline and vancomycin and resistance to kanamycin.

Of the strains identified as *B. cereus,* 5S, 11, 12, 14 and 17 had identical phenotypes based on substrate utilisation (Table 3). These strains could be distinguished from one another by their responses to the other antibiotics tested. Two other *B. cereus* strains, 1 and 9, were identical based on substrate utilisation. These strains produced acid from glucose, produced urease, protease, amylase, phospholipase C and were hemolytic. Strain 1 was resistant to ampicillin and sensitive to tetracycline, kanamycin, erythromycin, chloramphenicol, vancomycin and neomycin. Strain 9 differed from strain 1 in resistance to tetracycline and intermediate resistance to neomycin. The other three *B. cereus* strains had unique phenotypic profiles.

The two *B. weihenstephanensis* strains, 5 and 11S, differed from each other in the production of acid from sucrose and resistance to ampicillin. The *L. fusiformis* strain was negative for most tests except urease production (Table 3). The *E. faecalis* strains were unlike the *B. cereus* strains in that they did not produce enzymes for protease, amylase, and phospholipase C (Table 3). They differed from each other in their susceptibly to tetracycline, kanamycin and erythromycin.

Toxicity of pure cultures

The three *B thuringiensis* isolates (8, 18 and 18S), killed 100% of the *M. sexta* larvae as pure cultures. Because the crystal of *B. thuringiensis* is proteinaceous and is responsible for the toxicity, the LC_{50} was calculated from protein concentrations of whole cultures. The LC_{50} for the crystal-forming bacteria grown from soil sample 8 was 10.85 ng protein/diet pellet (7.23–15.2 ng protein/diet pellet 95% Confidence Limits, CL). For the strain 18S which was isolated from feeding soil suspension directly to *M. sexta* larvae, the LC_{50} was 35.4 ng/diet pellet (24.0–54.4 ng protein/diet pellet, 95% CL). For the strain grown from soil 18, the LC_{50} was 88 ng protein/diet pellet (64.1–117.3 ng protein/diet pellet, 95% CL). These concentrations are comparable to the LC_{50} of *B. thuringiensis* IBL 455 tested under the same conditions (56.1 ng protein/diet pellet, 45.7–68.9 ng protein/diet pellet 95% CL). There was no control mortality in any of these LC_{50} bioassays.

Of the other strains that fulfilled Koch's postulates, one *B. cereus* strain, 9, killed 100% of the *M. sexta* larvae tested using an undiluted culture at 8.5×10^7 spores/diet pellet. Based on titer, the LC_{50} was 6.0×10^6 spores/diet pellet. We did not perform LC_{50} bioassays on strains killing less than 100% of the larvae with an undiluted culture. Eight other *B. cereus* strains (1, 7, 11, 12, 14, 17, 19 and 21) killed greater than 50% of *M. sexta* larvae tested using an undiluted culture (average titer 5.0×10^8 cells/diet pellet). The pure undiluted cultures of *E. faecalis* and *B. weihenstephanensis* were less toxic killing between 18 and 43% of the larvae.

Discussion

Our results demonstrate, using microbial combinatorics, that insect larvae can be used to both select and enrich insect pathogens from combinations of bacteria

present in soil. This is best accomplished by amplifying all bacteria from an environmental sample on laboratory media prior to feeding the resulting mixture of cultured bacteria to larvae. This method selectively amplifies those bacteria able to grow under laboratory conditions and ensures the microbes grow *in vitro* and are pathogenic, as opposed to growing and being pathogenic only when present *in vivo*, as, for example, *Paenibacillus popilliae* Dutky (Klein 1986) against Japanese beetles or *Entomophaga maimaiga* Humber, Shimazu and Soper (Hajek 1999) against gypsy moth. Growth as a laboratory culture will also allow easier commercialisation of the pathogen.

Larvae that died following the consumption of these suspensions predominantly yielded bacteria consisting of a single colonial morphology. Nineteen of the bacteria thus recovered, re-fed to larvae, and reisolated, were identical, demonstrating that the bacteria initially recovered were responsible for larval mortality as per Koch's postulates.

As expected, after storage of soil for over a year, most of the bacteria recovered from the dead larvae formed spores. Three strains of bacteria were identified as *B. thuringiensis* by their ability to form crystals as well as their 16S rDNA sequence. Another 14 fell into the *B. cereus* group. Those spore formers killing greater than 60% of the larvae (1, 9, 15 and 17) often killed late at 96 h suggesting septicaemia rather than a toxin. Two spore-forming strains (5 and 11S) were identified as *B. weihenstephanensis*, a related species of bacteria, which previously has not been known to kill insects (Sorokin et al. 2006). *Lysinibacillus fusiformis* is also not known to be toxic to insects (Ahmed et al. 2007) although the related *L. sphaericus* (previously *B. sphaericus*) is known to kill mosquitoes (Charles, Nielsen-LeRoux, and Delécluse 1996). The extracellular gelatinase of an *E. faecalis* strain has recently been shown to affect the immune system in the greater wax moth (Park, Kim, Lee, Seo, and Lee 2007) and this may be similar to the toxicity of the *E. faecalis* strains we found in *M. sexta*. These *E. faecalis* strains had survived over a year in dry soil along with the spore forming bacteria, suggesting stability in the environment and demonstrating that combinatorics can discover novel pathogens.

Different species of bacteria were isolated from the same soil sample (5, 11 and 17) depending on whether the bacteria were isolated directly from the soil suspension or amplified on plates. These differences are probably due to the initial concentrations of the individual pathogenic bacteria in the soil and the ability of a strain of bacteria to replicate on the media chosen. The two *B. thuringiensis* strains isolated from soil sample 18 were not identical, suggesting that many pathogens could be isolated from an individual soil sample by varying conditions of growth and isolation.

The mode of action of the non-*B. thuringiensis* spore forming bacteria is not clear. We suspect that some act on the midgut because the visible sequence of events preceding death is similar to that associated with *B. thuringiensis* strains isolated in this study (Heimpel and Angus 1959). Other insect toxins such as the VIP (vegetative insecticidal proteins) toxins of *Bacillus* strains (Cao-Guo, Mullins, Warren, Koziel, and Estruch 1997), or toxin complexes (tc) of *Photorhabdus luminescens* Thomas and Poinar (Forst and Nealson 1996) also act on the midgut and have been identified in other bacteria (Hurst, Glare, Jackson, and Ronson 2000; Morgan, Sergeant, Ellis, Ousley, and Jarrett 2001). Other strains, such as 9, killed more slowly and did not show midgut damage (as evidenced by melanisation of the midgut) suggesting a true

pathogen rather than a toxin. Many of these strains produced proteases and phospholipase C which are known to enhance insect toxicity (Zhang, Lövgren Low, and Landén 1993).

The *M. sexta* larvae amplified, as well as selected, insect pathogenic bacteria from soil. The bacteria recovered from dead larvae were essentially pure cultures. Swiecicka, Bideshi and Frederici (2007) have recovered essentially pure cultures from *Trichoplusia ni* (Hüber) when fed a toxic strain of *B. thuringiensis.* The concentrations of a particular bacterial strain recovered from the larvae were higher than those for the same bacteria that occurred in the mixture fed to the larvae.

Direct soil suspensions, in general, were not as toxic to *M. sexta* larvae suggesting a low level of pathogens in the soil, as well as an absence of chemical pesticides in the soils tested. These bacteria were not abundant enough in the original soil to be distinguished from the background of other bacteria. The best-known bacterium for killing lepidopteran insect larvae such as *M. sexta*, *B. thuringiensis,* exists, on average, in soil at 10^3 spores/g wet weight soil (Martin 1995) and not all *B. thuringiensis* recovered from soil are toxic (Martin and Travers 1989). The LC_{50} for *M. sexta* 3rd instar larvae for a toxic *B. thuringiensis* is about 1000 spore equivalents (Martin, unpublished data). A 100 mg larva would have to consume 10 times its weight in a single dose of soil to eat enough spores to cause death. As *M. sexta* larvae do not usually consume soil, it is improbable that a larva could consume enough *B. thuringiensis* directly from soil to cause significant mortality. Spores of *B. thuringiensis* have also been recovered from the leaves of trees in quantities ranging from 28 spores/cm^2 on basswood to 980 spores/cm^2 on Norway maple (Smith and Couche 1991), again in quantities that do not cause epizootics but do occasionally cause insect mortality.

Microbial combinatorics directly isolates the desired end product: insect pathogenic bacteria. This process is counter-intuitive to the methods currently used to select potential bacteria for insect control (Ohba and Aizawa 1986; Travers et al. 1987). A selective process for isolation of *B. thuringiensis,* using a differential response to germination in acetate (Travers et al. 1987) requires growth for 4 h with vigorous shaking, heat treatment, overnight growth, selection of individual colonies, and growth for sporulation, before testing against insects. Soil samples taken in the same forest site using acetate selection in a prior study (Martin and Travers 1989), yielded 1016 bacterial isolates, which would require over 16,000 insects to screen for toxicity. In contrast, only 320 insects were used in the present study using similar soil samples by testing combinations of bacteria to establish initial toxicity. In addition, conventional methods only select spore-formers that survive the selection process, not necessarily those that are toxic to the insect. Another benefit of microbial combinatorics is that it requires only a single 48 h incubation period prior to testing bacteria against insects. The most significant advantage of microbial combinatorics over other selection methods is that at least one strain of bacteria from the test group is immediately known to be toxic. Microbial combinatorics relies heavily on the ability of a microbe with the desired characteristic(s) to successfully compete with other microbes from the same environment during amplification on laboratory media and in the insect.

Does this approach to the isolation of insect pathogenic bacteria have any utility beyond the model *M. sexta* system described? The three crystal-forming strains from soil samples 8 and 18 were also toxic to diamondback moth (*Plutella xylostella*

(Linnaeus) Lepidoptera: Plutellidae) larvae (R. Farrar, personal communication), a pest on cruciferous crops worldwide (Talekar and Shelton 1993). The same bacterial suspensions were toxic to larvae of another insect pest, Colorado potato beetle (*Leptinotarsa decemlineata* (Say) Coleoptera: Chrysomelidae), although speed-of-kill was slower than in *M. sexta* larvae. Multiple larvae had to be used to recover bacteria from these smaller insects (2 vs. 20 mg for *M. sexta* larvae). Although a predominant colony type was present, there was also a background of bacteria similar to those present in the larvae that died in the water controls. When grown in pure culture and fed back to *L. decemlineata* larvae, 41% of the larvae died, and weights of those surviving were significantly lower than the controls (2.5 ± 0.3 mg compared to 6.0 ± 0.58 mg in the controls, $P < 0.0001$). Thus, this process may be used to isolate insect pathogens directly from any insect that can consume bacteria.

The experiments described address only the simple case of a single strain of bacteria toxic to insects; future research will include investigation of mixtures of pathogenic bacteria to kill insects. Recent studies have examined mixed infection in insects (Martin 2002) and Raymond, Davis, and Bonsall (2007) suggested combinations of toxic and non-toxic bacteria are more effective. Our results also suggest that combinations of bacteria kill faster than a single bacterial strain at the same concentration. The mixtures of bacteria killed more quickly than pure cultures with the exception of the crystal forming isolates. Beyond isolation of bacteria from soil or other environmental sources to kill insects, this simplified combinatorics technique could be used to screen large culture collections. The Invasive Insect Biocontrol and Behavior Laboratory has a collection of over 40,000 strains of spore-forming bacteria (Martin and Travers 1989) that could be screened for insect toxicity. This technique is sensitive enough to pick out one toxic strain in a background of more than 150 non-toxic strains (Martin and Blackburn 2007).

Microbial combinatorics, testing bacteria in natural mixtures, can find novel insect pathogens. By changing the parameters such as growth media, time of growth and storage of soil or other source of bacteria, other pathogens may be favoured. It is also possible to use microbial combinatorics for directed selection of a pathogen for a specific target insect including newly introduced insect pests.

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